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ORIGINAL PAPER

Fecal progesterone and estradiol changes during the breeding season in captive female wolf

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Abstract: Understanding basic reproductive physiology is crucial for the management of both captive and free-ranging wolf. In the present study, we determined hormonal changes during pregnancy and the estrous cycle in captive female wolf by measuring fecal steroids collected during the breeding season with high performance liquid chromatography (HPLC). These biochemical analyses were validated using chemical devivatization and mass spectrometry, and interpreted along with the behavioral data. All four females undergoing estrus cycles were copulated with their partners and delivered pups successfully. We found that estradiol concentrations were significantly higher during the estrus cycle than other stages (p<0.01) and progesterone was also significantly increased throughout the pregnancy (p<0.01). These hormonal fluctuations demonstrated pregnancy-specific changes in the fecal progesterone and estradiol concentrations. Patterns of fecal estradiol and progesterone concentrations during estrous cycles were similar to those reported for other canids.

Keywords: *Canis lupus*; estradiol; estrus; fecal steroid; pregnancy; progesterone

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Introduction

Wolves (*Canis lupus*) are monogamous, seasonally monestrous animals. The estrous cycle of the species is usually from January to March (Mech 1970). Wolves in estrus copulate once a day and this period may continue three to five days (Young 1944; Murie 1944; Lentfer and Sanders 1973; Zimen 1976; Wasser et al. 1995; Velloso et al. 1998; Songassen et al. 2006). The gestation of wolves is about 62±4 days and their litter sizes vary from 1 to 11, with an average size at about six (Brown 1936; Woolpy 1968; Mech 1970; Lentfer and Sanders 1973; Lentfer and Sanders 1973). First breeders generally produce fewer pups than those with a breeding history (Rausch 1967; Lentfer and Sanders 1973).

Wolves in China were extinguished from much of their original range in the wild except Tibet, Sinkiang, Inner Mongolia and Northeast China (Zhang et al. 1999). Several studies on the wolves in China mainly focused on distribution, population density, anatomy, physiology, genetics, dens, reproduction and diet composition (Gao 2006). However, studies on reproductive physiology of wolves in China are scarce.

According to the growing interest in and concern about animal reproductive endocrinology, it is believed that the changes of some steroids in serum during the estrous cycle, such as progesterone, estradiol-17β and luteinizing hormone (LH), may have effects on the estrus behavior, vaginal smears and breeding success (Seal et al. 1979). There are some reports about these effects in canine animals also, such as the domestic dogs, wolves (*Canis lupus*), arctic foxes (*Alopex lagopus*) and coyotes (*Canis latrans*). Most data on relevant researches were obtained through studies of blood samples (Moller 1973; Smith et al. 1974; Concannon et al. 1975; Wildt et al. 1978; Seal et al. 1979; Wildt et al. 1979; Stellflug et al. 1981). However, because the blood samples of wild animals are difficult to obtain, and the collection process may affect the normal life of wild animals or even cause damage to them, more and more researchers turn to develop

non-invasive techniques to evaluate the animal conditions today, including biochemical analysis of urinary samples and fecal samples (Gudermuth et al. 1998; Sands and Creel 2004; Queyras and Carosi 2004; Ziegler and Wittwer 2005; Shimizu 2005; Walker et al. 2005). These methods were also introduced into the conservation biology researches. Several reports used the fecal and urinary hormonal assessments in evaluating the reproductive status of some canine animals, such as the researches in the maned wolves, *Chrysocyon brachyurus* (Gross 1992; Wasser et al. 1995) and the blue foxes, *Alopex lagopus* (Sanson et al. 2005). However, there is no report on the fecal and urinary hormonal assessment of wolves in China.

In the present study, in order to investigate the hormonal changes of wolves and to establish a rapid and efficient non-invasive method in the assessments of sex hormone levels changes of canine animals, we determined the progesterone and estradiol of the captive wolves throughout the whole breeding period by the biochemical quantitatively analyses of the fecal samples. This will be useful in evaluating the physiological status of wolves in breeding season and in researching the reproductive biological characteristics of other canine animals.

Materials and methods

Animals and study site

This study was carried out on four captive reproductive pairs of wolves (eight mature females and males) at Harbin North Forestry Zoo (45°31'N, 126°51'E) in Hei Longjiang Province, China. Each pair of the wolves observed in the present study inhabited in an enclosure consisting of a 20-m^2 outdoor area and a 2-m^2 indoor shed, respectively. The four females were named as F1, F2, F3 and F4.

Samples collection

For each female wolf, fresh fecal samples were collected 2–3 times per week during the breeding season at the early morning (05:30–09:00, from December 2005 to April 2006). Monitoring equipments were installed in this research. The fecal samples were collected with sterilized plastic bags at the defecate site and were then stored frozen at -20°C immediately until extraction and subsequence biochemical assays. Through monitoring, we can distinguish the exact source of fecal samples effectively.

Steroids extraction

The middle portion of the fecal sample were selected and put into a 50-ml Bunsen beaker, thawed at room temperature for about 10 min, dried in a heating oven until their mass was constant (about 4 h, 70°C). Dried fecal samples were well pulverized. Steroids were extracted using a protocol slightly modified from previously published methods (Brown et al. 1994; Wasser et al. 1994; Monfort et al. 1997; Touma et al. 2003; Turner et al. 2003). A total 0.5 g of the well-mixed powder was weighted in 5 ml capped plastic tubes, extracted with 1.5 ml double distilled water (ddH_2O) and 2.5 ml dichloromethane. The extracted samples were sonicated (5 min), vortexed for 2 h on a Multi-Pulse Vortexer. After centrifugation (20 min at 6 000 rpm), the dichloromethane layer (1 ml) was extracted and dried under nitrogen, then stored at -30°C until assay.

Chemicals and high-performance liquid chromatography (HPLC)

All steroids standards were purchased from Sigma Co (St. Louis, MO). HPLC grade acetonitrile (spectroscopically pure acetonitrile) was purchased from Tianjin Kermel Chemical Reagent Co., Ltd. Formic acid was analytical grade from Shanghai Chemical Reagent Co. Water was purified on a Milli-Q system (Millipore, Bedford, MA). The standard trichloroacetic acid (TCA, catalyst) for derivatization reaction at concentrations of 1.0% (v/v) was prepared by diluting the corresponding stock solutions (10%, v/v) of trichloroacetic acid with anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with P_2O_5.

HPLC separation of 2-(11H-benzo[a]carbazol-11-yl)-isopropyl-chloroformate (BCECH) derivatives was carried out by HYPERSIL–C18 column with a binary gradient elution, using the following linear gradient: Eluent A was 30% of acetonitrile consisting of 30 mM $HCOONH_4$ buffer (pH 3.5); B was acetonitrile (100%). During conditioning of the column and prior to injection, the mobile phase composition was 80% A and 20% B. The percentage of mobile was changed as follows after injection: 20%–45% (B) from 0 to 12 min; 45%–58% (B) from 12 to 15 min; 58%–100% (B) from 15 to 26 min; 100% (B) from 26 to 35 min. The flow rate was constant at 1.0 ml/min and the column temperature was set at 35°C. The fluorescence excitation and emission wavelengths were set at λex 275 nm and λem 380 nm, respectively.

Mass spectrometry

Experiments were performed using a LC/MSD-Trap-SL electrospray ion trap liquid chromatography/mass spectrometry (1100 Series LC/MSD Trap, a complete LC/MS/MS). All the HPLC system devices were from the HP 1100 series. Derivatives were separated on BDS HYPERSIL-C18 column (250 mm × 4.6 mm 5 μM, Yilite, China). The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI). The mass spectrometer system was controlled by Esquire-LC NT software, Version 4.1. A 35-min gradient elution (A: 30% acetonitrile consisting of 30 mM $HCOONH_4$, pH 3.5; B: 100% acetonitrile) was selected for the separation of fecal extracts derivatives. Fluorescence excitation and emission spectra were obtained at a 650-10 S fluorescence spectrophotometer (Hitachi). Excitation and emission bandpass were both set at 10 nm. The mobile phase was filtered through a 0.2 <mu>m nylon membrane filter (Alltech, Deerfiled, I L).

Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The FLD were calibrated and

tested using the FLD diagnosis procedure of the Chem-Station software for HP1100 system. The HP1100 LC/MSDSL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specification and sensitivity were achieved over the entire mass range.

Derivatization procedure and chromatographic separation

The target steroids derivatization were used the novel fluorescence reagent 2-(11H- benzo[a]carbazol-11-yl)-isopropyl-chloroformate (BCECH) and 2-(11H-benzo[a]carbazol-11-yl)-isopropyl-chloroformate (BCTCCL) as precolumn derivatization reagents for progesterone and estradiol, respectively. The BCECH-steroid derivatization proceeded in acetonitrile solution in the presence of trichloroacetic acid catalyst. The dried samples were added to 105 μL acetonitrile solution, and sonicated (5 min). A 100 μL of fecal extracts was added into a vial (1.0 mL), then successively added 80 μL of 5×10^{-3} BCEC acetonitrile solution and 30 μL of 1% trichloroacetic acid catalyst. The vial was then sealed and the mixture was heated at 65°C for 30 min in a thermostatic water-bath, and the reaction solution was cooled in ice-water to stop the reaction. An aliquot (10 μL) of the derivatization solution was injected to HPLC. The same procedure derivatization was performed on progesterone standards, and the calibration curves for each steroid-BCEC were obtained by linear regression plotting peaking area versus concentration.

The same procedure derivatization was performed on estradiol using BCTCCL except the mixture was heated at 65°C for 120 min in a thermostatic water-bath.

Separation of the derivative steroids was evaluated by the reversed-phase high performance chromatography. The standards utilized for this study included progesterone, testosterone, cortisol and corticosterone. Acetonitrile was used as mobile phase to give the optimal separation with the short retention. Chromatographic separation for four steroid compounds and estradiol are shown in Fig. 1 and Fig. 2, respectively. Although several impurity peaks were observed in the eluted procedure, no significant interference was generated.

Linearity, detection limits, recoveries and repeatability for steroids derivatives

The injection volume was 10 μL and the injected amount ranged from 100.0 pmol to 50.0 fmol (standard concentrations were 5.0×10^{-3}μmol/L – 10 μmol/L). The linear regression equations, correlation coefficients and detection limits were obtained as shown in Table 1. All of the steroids provided excellent linear responses, with correlation coefficients greater than 0.9998. The linear relationships at the higher concentrations were not tested. The corresponding detection limits for each steroids (at a signal-to-noise ratio=3:1) are 24.51–40.91 fmol. The recoveries were determined from the values obtained by the actual analysis of the fecal samples as calculated from the calibration graph constructed using the performed steroid derivatives. To three aliquot of 500 mg of feces, 10 μL of four steroid standards (1.0×10^{-4} mol/L) was, respectively, spiked into feces. The complete extraction and derivatization procedures were carried out according to the established method as described in experimental section. The analyses were carried out in duplicate. The experimental recoveries were between 90.4% and 105.4%. The repeatability of the method was ascertained by carrying out six assays on the same samples, each solution was injected twice. The values of the standard deviations for the retention time and peak area are showed in Table 1.

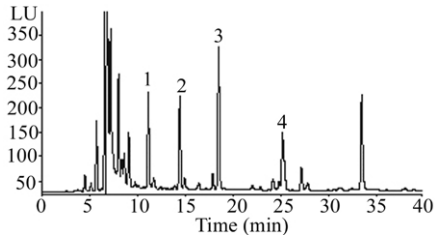

Fig. 1 Chromatogram of the derivatized standard steroid derivatives Chromatographic conditions as described in experimental section. 1. cortisol; 2. corticosterone; 3. testosterone; 4. Progesterone.

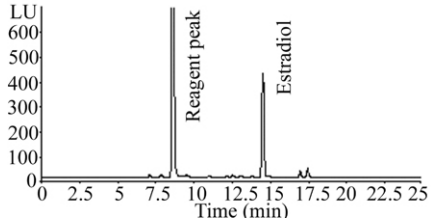

Fig. 2 Chromatogram of the derivatized standard estradiol derivatives. Chromatographic conditions as described in experimental section.

Table 1. Linear regression equations, correlation coefficients and detection limits for steroid derivatives and repeatability for peak area and retention time (n=6)

Steroids	Y=a*x+b	Regression coefficient (R)	Detection limits (fmol)	Retention time (RSD%)	Peak area RSD (%)
Estradiol	y=35.95x+12.14	0.9998	24.51	0.022	0.74
Progesterone	y=21.61x+12.47	0.9999	27.55	0.089	0.56
Testosterone	y=35.39x+10.90	0.9999	38.57	0.14	0.82
Cortisol	y=20.47x+13.38	0.9998	32.93	0.047	0.39
Corticosterone	y=21.57x+12.95	0.9999	40.91	0.067	0.29

Identification of steroid derivatives with APCI/MS

According to the optimal chromatographic conditions, the separation of steroid compounds for the extracted wolf feces is shown in Figs. 3–4. Although the chromatographic separation for real sample results in many impurity peaks, no interference was observed for the separation of all steroids derivatives as they were eluted in the time range of 10–25 min. Identification of derivatives was simultaneously carried out by the method of standard addition and MS analysis.

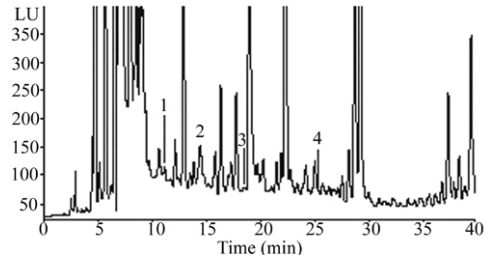

Fig. 3 Chromatogram of steroids from the extracted wolf feces. Chromatographic conditions and peaks as Fig. 1.

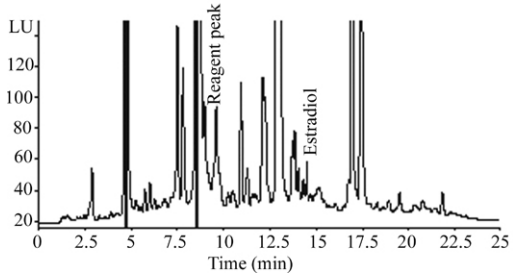

Fig. 4 Chromatogram of estradiol from the extracted wolf feces. Chromatographic conditions and peaks as Fig. 2.

Statistical analysis

All fecal data are expressed on a per gram dry feces basis. We calculated the concentration of fecal metabolites (pg/g of feces) in every sample. When it is necessary, data are presented as the mean ± standard error of the mean. We separated the whole survey period as pro-estrus, estrus, and gestation. The individual values in each part were averaged for each wolf and for the whole group. We analyzed and compared how these mean values changed throughout the whole period for individuals or as a whole, simultaneously. Analyses were conducted using one-way analysis of variance (ANOVA). Differences were regarded as significant if $p<0.05$ and as highly significant if $p<0.01$. Statistical software package SPSS version 13.0 (SPSS Inc.) was used for all the tests.

Results and discussion

For the four females, F1 delivered four pups on April 9, 2006; while F2, one pup on April 16, 2006; F3, six pups on April 8, 2006; F4, six pups on April 3, 2006, respectively. The fecal estradiol and progesterone concentrations profile of the four female wolves in the whole breeding season were shown in Fig. 5-6, respectively.

The F1 came into pro-estrus in the mid of January, 2006 and had mating behavior with its partner from January 29, 2006 to February 8, 2006. The mean concentration of the fecal estradiol was 54.3±14.9 pg/g in pro-estrus, whereas, the value was 129.8±13.7 and 69.5±7.2 in estrus and gestation, respectively. Fecal estradiol concentrations in estrus are significantly higher ($p<0.01$) than that in pro-estrus and pregnancy. But the difference between pro-estrus and pregnancy is not significant ($p>0.05$). At the copulation period the female wolf had an estradiol concentrations peaking at 266.2 pg/g on January 29, 2006. After that, the estradiol concentrations fluctuated from 40.0 pg/g to 80.0 pg/g throughout gestation. The mean concentrations of the fecal progesterone of this female wolf were 347.4±50.5, 511.2±56.1, and 635.9±55.9 pg/g on pro-estrus, estrus and gestation, respectively. Difference between pro-estrus period and pregnancy was highly significant ($p<0.01$). Progesterone increased from the time of the estradiol peak and achieved its highest concentration on January 20, 2006, (1 245.6 pg/g), while estradiol was 266.1 pg/g. Progesterone then declined gradually and irregularly during pregnancy to 765.7 pg/g on April 3, 2006, six days before parturition.

The wolf F2 had a fecal hormone pattern similar to that of F1. It had mating behavior with its partner from February 5, 2006 to February 8, 2006. The Estradiol concentrations of this wolf was significantly higher ($p<0.01$; 155.5±25.7 pg/g) in estrus than pro-estrus (40.5±5.6 pg/g). It had elevated estradiol concentrations several days before copulation, peaking on January 28, 2006 at 469.2 pg/g, and then fluctuated between 80.0–250.0 pg/g until copulation finished when estradiol declined below 80.0pg/g. It held a low and relatively constant estradiol concentration throughout pregnancy. A high progesterone peak (2 512.7 pg/g) was observed on January 28, 2006, about five days before copulation. Progesterone concentrations in estrus were higher ($p>0.05$; 578.2±43.6 pg/g) than that in pro-estrus (462.8±67.5 pg/g).

The wolf F3 had mating behavior with its partner from January 27, 2006 to February 8, 2006. It exhibited patterns and relationships between estradiol and progesterone concentrations similar to those of F1 and F2 to some extent. Two estradiol peaks of 256.8 pg/g and 253.7 pg/g occurred on January 25, 2006 and February 9, 2006, respectively. Estradiol concentrations were less than 40.0 pg/g during the non-breeding season, while the mean concentration was 20.7±7.8 pg/g. At the end of estrus, estradiol declined gradually and slowly. But the decline was not significant ($p>0.05$) compared with the estrus period. Progesterone concentrations were significantly ($p<0.05$) higher in breeding season than in non-breeding season. A progesterone peak of 3 283.3 pg/g occurred on January 22, 2006, four days before mating behavior started. It then fluctuated irregularly until pregnancy. Average progesterone concentration was 1 011.8±159.6 pg/g during pregnancy. In the lactation, progesterone was approximately 2.5 times lower (954.3 pg/g) than the peak concentration.

The F4, which had mating behavior with its partner from January 29, 2006 to February 3, 2006, had a fecal hormone patterns similar to that of the above animals on estradiol and progesterone. Differences among the three periods on the survey of its estradiol concentrations in feces were highly significant ($p<0.01$). The mean concentrations of fecal estradiol were 51.8±15.0, 199.8±31.5, and 79.3±6.8 pg/g in the pro-estrus, estrus and gestation, respectively. Estradiol concentrations rose during the pro-estrus period and achieved its highest concentra-

tions at 533.1 pg/g on January 29, 2006. After copulation, estradiol concentrations declined to a low concentration at 161.0 pg/g abruptly, and then fluctuated between 50.0–70.0 pg/g during the pregnancy. Progesterone concentrations fluctuated irregularly until peak at 2 512.7 pg/g on January 25, 2006, then declined abruptly to a low concentration and fluctuated below 1,000.0 pg/g during the gestation.

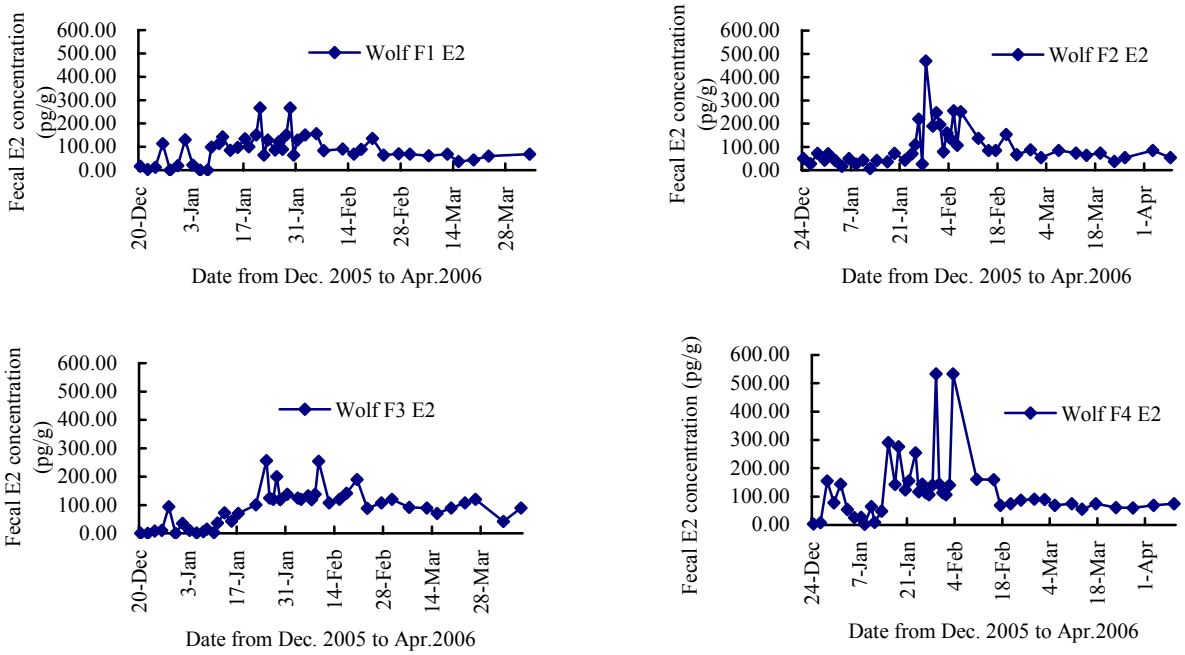

Fig. 5 Individual profiles of fecal estradiol (E2) concentrations in captive female wolves F1-F4

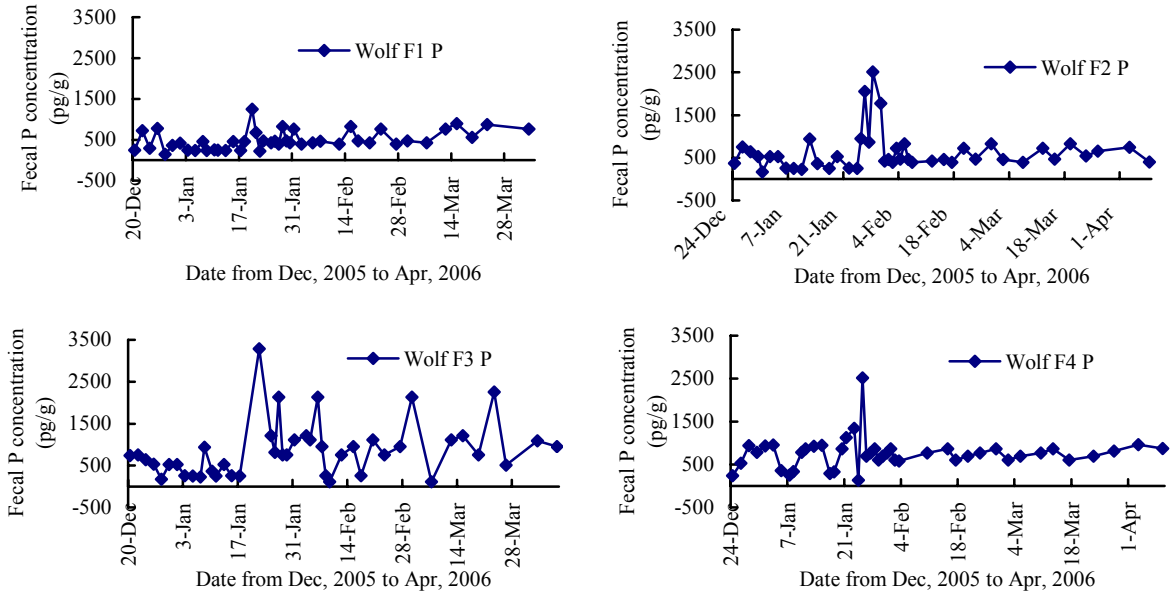

Fig. 6 Individual profiles of fecal progesterone (P) concentrations in captive female wolves F1-F4

Based on the analyses of serum steroid, the changes of reproductive hormones in female wolves during the breeding season were already studied (Seal et al. 1979). However, some other reports in canine animals, such as domestic dog (Gudermuth et al. 1998), maned wolf (Songsasen et al. 2006), African wild dog (Lycaon pictus, Monfort et al. 1997) and blue fox (Sanson et al. 2005), showed the usefulness of fecal steroid in evaluating the concentrations of estradiol and progesterone. In the present

study, we attempted to evaluate the level of sex hormones in female wolves during the breeding season by using the biochemical analyses of fecal samples. According to our results, there were significant differences in the fecal excretory patterns of ovarian steroids among different stages of breeding season. Overall fecal estradiol concentrations were significantly higher in estrus than in pro-estrus and gestation while fecal progesterone concentrations in the other two periods of wolves were significantly higher than in pro-estrus. This pattern of fecal steroids excretion is similar to the results of previous reports of wolves (Seal et al. 1979) which were based on serum steroid analyses and also similar to the findings in bitch (Gudermuth et al. 1998) and blue fox (Sanson et al. 2005). This means that ovarian progesterone is necessary to maintain pregnancy of wolves as it is in other canine species (Moller 1973).

The analyses of the changes in hormone levels combined with the mating activity of wolves indicate that, before mating, estradiol concentration reached the peak and the progesterone concentration also increased, especially 24 h or more earlier. About 24−48 h after the peak values of estradiol reached and progesterone increased, the copulatory behavior occurred. This indicates that estradiol and progesterone are both important factors for promoting the female's mating activity. The copulation couldn't occur when estradiol reached the peak without progesterone concentration increase.

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